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Enhanced Degradation of the Fungicide Vinclozolin: Isolation and Characterisation of a Responsible Organism

Ronald B. Cain* & Jean A. Mitchell‡

Environmental Microbiology Laboratory, Department of Biological & Nutritional Sciences, The University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU, UK

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Abstract: Enhanced degradation of the fungicide vinclozolin was stimulated by multiple successive applications to a soil without any history of previous pesticide input. A vinclozolin-degrading bacterium isolated from this soil was identified as a strain of *Pseudomonas putida*. This organism metabolised vinclozolin as a source of carbon, but it would neither grow with nor transform any other closely related dicarboximide fungicides nor the degradation product, 3,5-DCA. The degradation of vinclozolin by cultures of *P. putida* St-1 was investigated under various culture conditions; biodegradation was optimal at 23°C, pH 6·5 and inoculum densities of 10^7 cells ml^{-1} but cultures would grow from as little as 100 cells ml^{-1}. Amendments of the vinclozolin-degrading isolate to soil previously untreated with the fungicide caused rapid degradation of applied vinclozolin, whereas amendments of boiled cells, or viable cells grown in the absence of vinclozolin, produced no discernible effect on the rate of vinclozolin degradation.

Key words: vinclozolin, biodegradation, *Pseudomonas putida*, enhanced degradation, 3,5-dichloroaniline, microbial populations in soils, oxazolidine ring metabolism

1 INTRODUCTION

Failure in the mid-1970s in the field performance of the soil-applied herbicide EPTC[1] and the insecticides bufencarb and carbofuran[2] was attributed to their enhanced degradation. The importance of this phenomenon as a potentially major cause of compromised pest control is now widely acknowledged for several other pesticides, fungicides and herbicides.[1–4]

While many pesticides undergo enhanced degradation in soil, only in some instances have specific microorganisms with the ability to degrade the compounds *in vitro* been characterised; such pesticides include carbofuran,[5] EPTC,[6] isofenphos[7] and diazinon.[8] The dicarboximide fungicides vinclozolin and iprodione, used commercially for the control of many crop foliage diseases, but especially in the UK as a soil drench or granular application for onion white rot, are known to be subject to enhanced degradation after repeated application to soil;[4,9] the agent was suspected to be microbial, although the abiotic instability of these dicarboximides in alkaline field soils undoubtedly contributed to their lack of persistence.[5,9,10]

The experiments described here were designed to isolate, characterise and identify the putative organism(s) responsible for the enhanced biodegradation of vinclozolin in a British soil, to demonstrate the ability of a pure culture of the organism(s) to degrade the fungicide *in vitro* and to determine the optimum conditions for such degradation before ensuring that the isolated organism(s) could degrade vinclozolin when returned to untreated soil.

2 MATERIALS AND METHODS

Analytical grade and technical grade vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-oxazolidine-2,4-dione]

* To whom correspondence should be addressed.
‡ Present address: Acer Environmental, Howard Court, Manor Park, Daresbury, Runcorn, Cheshire, WA7 1SJ.

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were provided by BASF Ltd, Ipswich, Suffolk, UK. Other formulated fungicides, as used agriculturally, were gifts from Mr D. L. Suett and Dr A. Walker of Horticulture Research International, Wellesbourne, Warwicks., UK. Formulated vinclozolin (1) and iprodione (2) were commercial wettable powders (ronilan and rovral, respectively), containing approximately 500 g kg^{-1} of the active ingredient (AI). Formulated procymidone (6) contained 500 g AI kg^{-1} and myclozolin (4) 330 g AI kg^{-1}. Analytical grade 3,5-dichloroaniline (3,5-DCA) (5) was purchased from Aldrich Chemicals UK (Fig. 1).

2.1 Soil experiments

Soil used in the laboratory studies of vinclozolin degradation was obtained from field plots, with no known history of treatment with any pesticide, herbicide or fungicide for at least 35 years, at the experimental gardens of Newcastle University at Close House, Heddon-on-the-Wall, Northumberland. This soil has been described previously.[10] Soil taken by core sampler from the top 10 cm of field plots was dried to 20% (w/w) moisture content and passed through a sieve with 2-mm mesh; 50 g was placed in each of six glass dishes (5 cm deep × 9·5 cm diam.), which were stacked one above the other in a closed glass container to maintain constant moisture conditions. To induce enhanced degradation, dishes of soil were treated with an aqueous suspension of formulated vinclozolin to give a final concentration of 5 μg AI g^{-1} dry weight of soil and the contents intimately mixed; the dishes were then incubated at 25°C for two weeks, by which time fungicide residues had disappeared. The moisture content of the dishes was kept constant by adding distilled water to the dishes every two days to return them to their original weight. The fungicide treatment was repeated up to three times at similar two-week intervals. Soil subjected to these pretreatments was checked for the absence of vinclozolin residues before it was used in subsequent experiments; if traces were detected, incubation was continued until they had disappeared. Control (untreated) soils were treated with equivalent volumes of water instead of fungicide, but were otherwise subjected to an identical incubation regime.

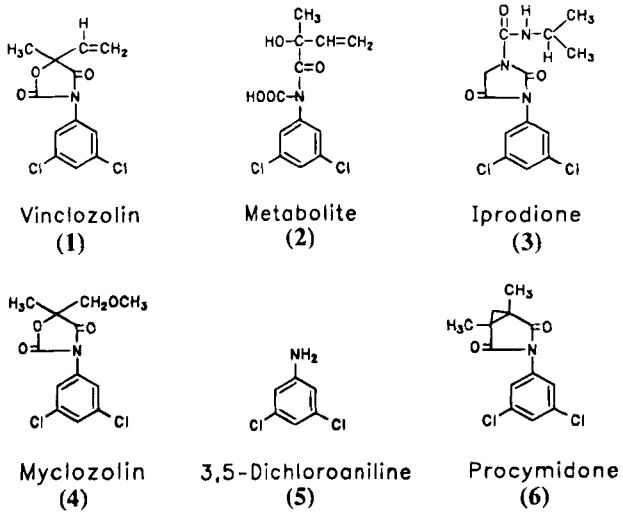

Fig. 1. Structures of vinclozolin and some of its analogues.

For subsequent experiments, formulated vinclozolin was incorporated into samples of vinclozolin-pretreated and untreated soil at approximately the manufacturer's recommended field dose, calculated as 25 μg AI g^{-1} dry weight of soil. The dishes of soil were then incubated at 23°C in the dark. Samples were taken and analysed for residual fungicide using HPLC as previously described.[10]

2.2 Culture media

The mineral salts medium used for enrichments and pure culture isolation comprised: KH_2PO_4, 1; $(NH_4)_2SO_4$, 0·1; $CaSO_4$, 0·05; $MgCl_2.6H_20$, 0·05 g $litre^{-1}$ and trace elements solution, 1 ml $litre^{-1}$. The trace elements stock solution comprised: $FeSO_4.4H_2O$, 400; $MnSO_4.4H_2O$, 400; $ZnSO_4.7H_2O$, 200; $CuSO_4.5H_2O$, 40; $CoCl_2.6H_2O$, 40; KI, 300 and $Na_2MoO_4.2H_2O$, 50 mg $litre^{-1}$ glass distilled water. The salts were dissolved in glass double-distilled water, adjusted to pH 6·5 with KOH (2 M) and sterilised at 121°C and 103 kPa steam pressure. When required, this medium was solidified with agar (Oxoid No. 3; 15 g $litre^{-1}$). Thermolabile carbon sources (including vinclozolin) were added to cooled, previously sterilised medium from filter-sterilised stock solutions.

In some experiments the mineral salts addition was supplemented with yeast extract (5 g $litre^{-1}$) and Bactopeptone (5 g $litre^{-1}$) (YXBP medium) or with soil extract (100 ml $litre^{-1}$) (MSSE medium). Soil extract was prepared[11] from Close House soil; analysis revealed that when finally constituted into the mineral salts medium, it added 0·7 mg $litre^{-1}$ of available ammonia, 0·3 mg $litre^{-1}$ of available nitrate and 24·7 mg $litre^{-1}$ of total organic carbon to the normal constituents of the medium.

2.3 Isolation and identification of fungicide-degrading organisms

Pretreated soil (5 g), shown experimentally to be active in degrading vinclozolin, was added to sterile phosphate buffer, (60 mM, pH 7·5; 50 ml) and macerated (2 min, 1500 rev min^{-1}) in a Waring blender. A series of 1 : 10 dilutions of the suspension was made in the phosphate buffer and aliquots (1 ml) of the dilutions between 1/100 and $1/10^8$ were used to inoculate 100-ml Erlenmeyer flasks containing sterile mineral salts medium (20 ml) with analytical grade vinclozolin at 0·1 mM. After incu-

bation for five days at 23°C and 200 rev min^{-1}, the flask contents were analysed for residual fungicide. The contents of flasks which showed evidence of substantial fungicide degradation were subcultured (0·05% (v/v) inoculum) into fresh liquid medium with the fungicide as the sole source of carbon and energy. Several such mixed cultures derived from separate enrichments of vinclozolin-pretreated field soil from Close House were maintained in liquid culture by repeated subculture in the same liquid medium at 10- to 11-day intervals. Pure isolates were obtained from the greatest dilutions of the macerate of soil and from serially diluted mixed cultures, by repeated subculture (0·1% (v/v) inoculum) into liquid medium in which the fungicide was the sole source of carbon and energy, i.e. by dilution to viable extinction. Once obtained, putative pure strains were maintained at 25°C by weekly passage in liquid mineral salts medium containing soil extract plus vinclozolin at 0·1 mM.

Incorporation of soil extract into liquid media had no significant effect on the observed rates of vinclozolin utilisation but it increased colony size on solidified mineral salts medium, it significantly prolonged the viability of pure cultures and it reduced the frequency of unpredictable spontaneous loss of vinclozolin-degrading ability in pure cultures. During the course of the investigation, pure cultures of several vinclozolin-degrading strains (e.g. isolates A, F, St-1) were obtained by these techniques on numerous separate occasions, but most were eventually discarded when they spontaneously lost their degrading ability during repeated subculture in liquid minerals salts medium containing vinclozolin. One isolate, St-1, had a particularly stable vinclozolin-degrading ability and was used in most of the experiments. This vinclozolin-degrading pure isolate was examined for its biochemical activities using API 20N and API 20NE rapid identification kits (API System, La Balme les Grattes, 38390 Montalieu-Vercieu, France). The DNA base composition of the isolated organism was determined semi-quantitatively by using a modification of the method of McMeekin[12] which compared the UV sensitivity of the fungicide-degrading organism and *Pseudomonas putida* PaW-1 (NCIMB 10432; National Collection of Industrial and Marine Bacteria, 23 St Machar Drive, Aberdeen, AB2 1RY, UK). Nutrient agar plates were spread with 0·1 ml of a stationary phase culture of either *P. putida* PaW-1 or the isolated bacterium adjusted to the same cell density. Different areas of the plates were then exposed to UV radiation from a UV transilluminator (UV Products Ltd, San Gabriel, CA, USA) by placing the open plates, covered by a UV-opaque plastic sheet, face down on the transilluminator. The plastic sheet was then progressively moved exposing sequential fresh sections of the plate to radiation. After three days' incubation in the dark, the plates, including unirradiated controls, were examined and inhibition of growth compared.

2.4 Degradation of vinclozolin by pure cultures of bacteria

Triplicate Erlenmeyer flasks (100 ml) containing mineral salts medium (25 ml) with the analytical grade compound (0·05–0·25 mM) as source of carbon and energy were inoculated (4% (v/v)) from a stock culture of the pure strain of vinclozolin-degrading bacterium. Triplicate flasks prepared in the same way but with the addition of sodium azide (0·1% (w/v)), were also included in the incubation, along with uninoculated control flasks. The cultures were incubated in the dark at 23°C and sampled (0·65 ml) for viable counts and residual vinclozolin over the time course of the experiments.

The effect of temperature on vinclozolin degradation was examined in flasks incubated in a refrigerated incubator shaker at a range of temperatures (4, 15, 23, 30, 37 and 42°C). Because experiments with soil had indicated that pH was also important in fungicide degradation,[13] liquid media were adjusted to pH 5·5, 6·0, 6·5, 7·0 or 7·5 by the addition of NaOH (0·1 M) or HCl (0·1 M) where necessary, with the final pH checked using a glass electrode. Inhibitory effects in liquid culture of 3,5-DCA, a major metabolite of vinclozolin,[9,10] were examined by adding 3,5-DCA (0–100 $\mu g\ ml^{-1}$) to liquid cultures. Carbon sources (fructose, glucose, galactose, mannose, xylose or starch) were added (10 g $litre^{-1}$) to some flasks to examine the effect of alternative carbon sources on rates of vinclozolin degradation. Additional nitrogen was also added as ammonium nitrate (1–10 g $litre^{-1}$) to some cultures. Media containing soil extract,[11] with vinclozolin as the principal source of carbon and energy, were inoculated to give final concentrations of cells of 10^4–10^8 cells ml^{-1} of media. The degradation of vinclozolin was monitored by HPLC until it had disappeared or the residual amount remained constant, at which time the number of viable cells in the cultures was redetermined by viable counting on YXBP and on MSSE media, both containing vinclozolin (0·1 mM). Total counts were made by direct counting in aliquots of an appropriately diluted culture in a haemocytometer with Thoma ruling, using a phase-contrast microscope.

2.5 Metabolism of compounds by cell suspensions of the pure isolates

Late exponential phase or early stationary phase cells of strains F and St-1, grown on MSSE medium plus 0·1 mM vinclozolin, were harvested, washed and resuspended in mineral salts medium to give approximately 1 mg dry weight cells ml^{-1} (A_{540} approx. 2). Aliquots of cell suspension (10 ml) were dispensed into triplicate 100-ml Erlenmeyer flasks. Vinclozolin (**1**) and its analogues or metabolites (3–6) (Fig. 1) were added from filter-sterilised concentrated stock solutions in

aqueous acetone to give a final concentration of 0·25 mM based on the active ingredient (maximum concentration of acetone in final solution was <0·5 ml litre^{-1}). The experimental flasks containing substrates and active cell suspensions were incubated at 23°C with shaking (200 rev min^{-1}) for the duration of the experiment. Control flasks, containing boiled cell suspensions or no cells, were also included. Supernatants were analysed by HPLC; the system used[10] resolved all the test compounds from each other and from their hydrolysis and metabolic products.

2.6 Microbial amendments to fungicide-treated soils

Cultures (3 × 500 ml) of the vinclozolin-degrading St-1 strain, isolated from Close House soil, were grown up for 14–16 h in YXBP broth supplemented with vinclozolin at 50 μg ml^{-1}, and harvested by centrifugation at 4000 g. The washed cells were pooled, rewashed and suspended in mineral salts medium and then added to replicate dishes each containing 50 g of untreated or vinclozolin-degrading soil[10] to give final densities of the microbial supplement of none or 10^4–10^8 cells g^{-1} dry weight of soil. Boiled cells (10^8 cells g^{-1} dry weight of soil) were added to replicate control dishes of both types of soil. The same organism, but grown in YXBP medium without vinclozolin, was also used for soil amendment at 10^8 cells g^{-1} dry weight of both types of soil. The cell suspensions were adjusted so that the same volume of mineral salts medium was added to all dishes. For the 'no cell' control, mineral salts medium only was added. The soil dishes, adjusted to constant moisture level (20% (w/w)) were stacked in the glass containers and were incubated at 23°C in the dark for 24 h, after which time formulated vinclozolin (25 μg AI g^{-1} dry weight of soil) was incorporated into all soil dishes, which were then reincubated, sampled and analysed for fungicide residues over the next few days.

2.7 Treatment of data

The calculation of the biodegradation constants ($-k_D$) for vinclozolin in this paper are corrected for abiotic loss, but the statistical treatment of the analytical data[14] was the same as described in the previous paper.[10]

3 RESULTS

3.1 Laboratory-based soil experiments

In field soil treated with vinclozolin on three previous occasions, degradation of 25 μg vinclozolin g^{-1} dry weight soil was extremely rapid at 23°C and 20% (w/w) moisture content, and after 56 h less than 10% of the initially available fungicide remained (Fig. 2). In previously untreated soil there was negligible fungicide loss

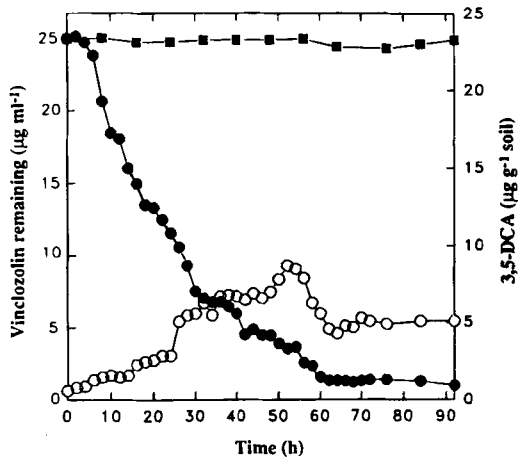

Fig. 2. Degradation of vinclozolin in laboratory incubations by (●) field soil treated on three previous occasions with the fungicide and (■) field soil never previously exposed to the fungicide. (○) 3,5-Dichloroaniline accumulated from added vinclozolin in the soil previously exposed to vinclozolin.

over 96 h. The appearance of 3,5-DCA was observed in pretreated soils during fungicide degradation, though it did not accumulate stoichiometrically with fungicide disappearance. Pretreated soil from this experiment, and from other field plots similarly pretreated,[10] was used as the source of bacteria for enrichment cultures.

3.2 Enrichment and isolation of vinclozolin-degrading organism

Initial attempts to obtain organisms with the ability to degrade vinclozolin using conventional elective shake flask cultures inoculated with vinclozolin-pretreated soil, followed by streaking loopfuls of the culture medium after 5–20 days growth on vinclozolin-supplemented mineral salts or MSSE agar, were unsuccessful. Though fungicide did disappear in the enrichment flasks, this property was unstable and many enrichment cultures spontaneously lost their degradative capacity after several (sometimes more than 10) subcultures in mineral salts medium plus vinclozolin. Inoculating well-separated micro-colonies from plates of mineral salts plus vinclozolin agar back into the corresponding liquid medium did not yield cultures which could degrade vinclozolin. Vinclozolin-degrading mixed cultures were also established from serial dilutions (1/100 to 1/10^8) of soil macerates from vinclozolin-pretreated soils. In some of these cultures also, spontaneous loss of vinclozolin-degrading ability occurred, but it was less frequent in cultures containing soil extract plus fungicide than in those based solely on mineral salts with fungicide. pH had a marked effect on the outcome of enrichment procedures because vinclozolin is particularly base-labile above pH 7·5[15] and at pH values below 5·5 its biodegradation in soil dishes[10] or in mixed cultures derived from such soil[5] was significantly

retarded. Successful mixed cultures, with persistent ability to degrade vinclozolin through repeated subculture, were best achieved at pH 6·5 with the soil extract supplement.

Pure fungicide-degrading isolates were obtained by repeated subculture using small inocula, at increasing serial dilutions, into media containing only the fungicide as carbon source until, on a statistical basis, only a single organism was present in the culture at inoculation; i.e., dilution to viable extinction. Using this tedious dilution technique, several pure isolates were obtained during the investigation and were used in some experiments but many of these eventually lost their vinclozolin-degrading ability before they had been identified. One particularly stable isolate, strain St-1, was eventually obtained and used for the experiments described in this paper. This culture was checked for purity by plating samples on MSSE and YXBP media where it formed whitish translucent, entire, smooth colonies, 1–3 mm diameter after three to five days at 25°C. On mineral salts agar containing 0·1 mM vinclozolin only pin-point colonies (0·1–0·2 mm diam.) resulted after seven days at 25°C and these would not re-establish growth in liquid medium of the same composition. The bacteria from colonies on MSSE medium were short (3 μm × 1 μm) Gram-negative rods, vigorously motile by polar flagella. They were oxidase and catalase-positive. When the DNA composition of the isolates was examined by UV sensitivity, the vinclozolin-degrading pure isolate had almost identical sensitivity (total kill after 15 s) to short-wave UV radiation as *Pseudomonas putida* PaW-1, the (% G + C) composition of which has been measured as 60·7(±0·5)%.[16] The results from the API System test kits of 16 biochemical tests, 14 carbon source utilisation tests and nine carbohydrate oxidation tests completed on the vinclozolin-degrading strain and on *P. putida* PaW-1 were identical. The strain also gave strong pigmented growth on King's medium and utilised the appropriate unusual carbon sources that distinguish *P. putida* from other pigmented pseudomonads,[25] strongly suggesting that the vinclozolin-degrader was a strain of *Pseudomonas putida*.

3.3 Vinclozolin degradation by *Pseudomonas putida* in liquid cultures

Pure cultures of *P. putida* strain St-1, grown from inocula of 10^2–10^6 cells ml^{-1}, all degraded vinclozolin (0·25 mM) completely within 120 h in mineral salts medium at pH 6·5, 23°C and an orbital shaker speed of 180 rev min^{-1}, attaining a population density of 2·2 × 10^7 cells ml^{-1} (Fig. 3). Under these conditions the specific growth rate, μ, was 0·013 h^{-1} (t_d approx. 51 h). The time taken for total disappearance of 0·05 mM vinclozolin at 23°C was about 100 h, though at 35°C it was

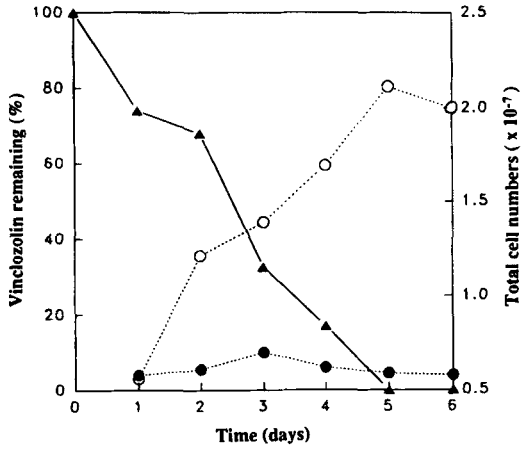

Fig. 3. Growth of *Pseudomonas putida* St-1 in mineral salts medium containing 0·25 mM vinclozolin as sole source of carbon and energy. Residual vinclozolin (▲) and total cell numbers in cultures (○) containing vinclozolin or (●) without vinclozolin.

less than 48 h (data not shown). The addition of sodium azide (1 g litre^{-1}) to growing cultures reduced its degradation rate to that observed in uninoculated control flasks (see Fig. 5(a)).

A culture of *P. putida* St-1, grown in media containing vinclozolin as the sole carbon source, was harvested, the cells washed twice in cold phosphate buffer to remove traces of the fungicide and used to inoculate flasks of media containing vinclozolin (0·05 mM) as a limiting source of carbon, to give initial densities of organisms between zero and 10^8 cells ml^{-1} medium. Media inoculated with the greatest cell densities displayed the most rapid degradation of vinclozolin but the increase in degradation rate with increase in inoculum size plateaued at 10^7 cells ml^{-1} (Fig. 4). At this cell density the maximum rate of degradation ($-k_D$ 3·62 day^{-1}) gave 90% loss of the available vinclozolin in 2.3 days. Denser inocula did not further increase the rate of vinclozolin degradation. At the end of the experiment

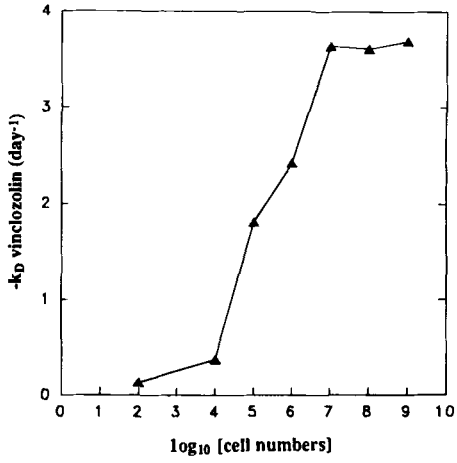

Fig. 4. The effect of inoculum size of *Pseudomonas putida* St-1 on the degradation constant for vinclozolin in cultures grown in mineral salts medium containing 0·05 mM vinclozolin.

(10 days) all the flasks with initial inoculum densities between 10^3 and 10^6 cells ml^{-1} contained viable cell numbers between 3×10^6 and 7×10^6 (mean $5{\cdot}2 \times 10^6$) cells ml^{-1} of culture media, indicating that 0·05 mM vinclozolin as limiting C-source supported this density of cells. In a similar experiment 0·25 mM vinclozolin had supported $2{\cdot}2 \times 10^7$ viable cells ml^{-1} (Fig. 3), so there was good agreement between the mean population densities attained at the two vinclozolin concentrations.

The major metabolite of the degradation of vinclozolin was 3,5-DCA, which accumulated in cultures to approx. 8·3 μg 3,5-DCA ml^{-1} from an initial 15 μg vinclozolin ml^{-1} (Fig. 5(a)); this is 98% of the calculated stoichiometric conversion. No other UV-absorbing metabolites were noted, even transiently, in cultures. The initial rate of biodegradation of vinclozolin by pure

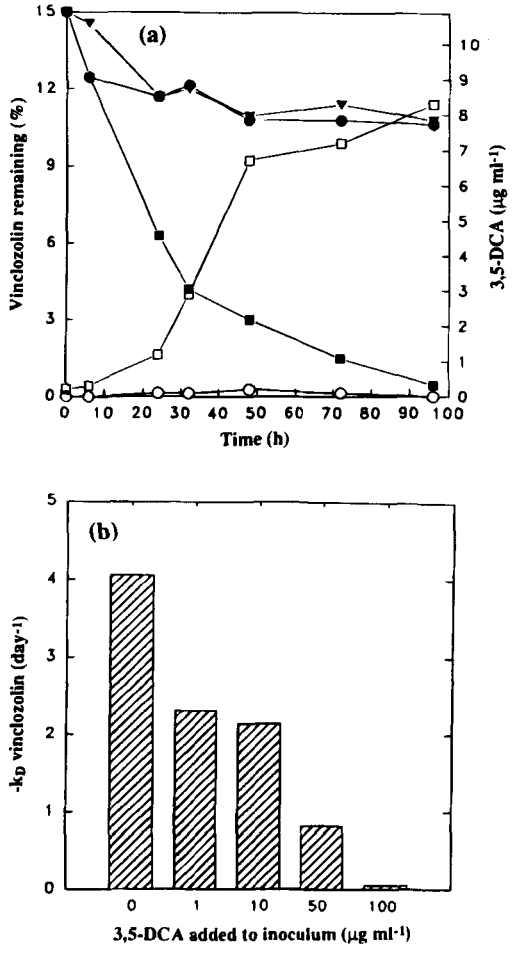

Fig. 5. (a) Accumulation of 3,5-DCA from vinclozolin (15 μg ml^{-1}) in mineral salts medium at pH 6·5 and 23°C, by *Pseudomonas putida* St-1. Concentrations of (■) vinclozolin and (□) 3,5-DCA in inoculated flasks; (▼) vinclozolin in uninoculated control flasks, and (●) vinclozolin and (○) 3,5-DCA in inoculated cultures inhibited with sodium azide. (b) The effect of 3,5-DCA, when added with the inoculum, on the degradation constant ($-k_D$) for vinclozolin in cultures of *P. putida* St-1 growing with 0·05 mM vinclozolin in MSSE medium at pH 6.5 and 23°C.

cultures of *P. putida* St-1 was markedly diminished, however, by the presence at inoculation of as little as 1 μg 3,5-DCA ml^{-1}, and 100 μg 3,5-DCA ml^{-1} caused almost complete inhibition of vinclozolin biodegradation (Fig. 5(b)). In uninoculated media controls, 3,5-DCA did not affect the abiotic rate of vinclozolin disappearance (data not shown).

There were considerable differences in vinclozolin degradation rates between culture media incubated at different temperatures; at 30°C and above, rapid degradation occurred in inoculated, and was measurable in uninoculated, cultures (Fig. 6). Biodegradation in cultures, corrected for abiotic losses was, however, temperature-dependent but inhibited by extremes of temperature (<5°C and >40°C). Vinclozolin was noticeably decomposed abiotically in mineral salts or MSSE media at temperatures of 37°C ($-k_D$ 0·213 day^{-1}) and above.

The overall rate of vinclozolin degradation increased with pH between 5·5 and 7·5 in both inoculated and uninoculated cultures. The optimum for vinclozolin biodegradation, corrected for abiotic loss, was pH 6·5 ($-k_D$ 5·60 day^{-1}) but significant biodegradation still occurred at pH 5·5 (k_D 3·45 day^{-1}) in inoculated cultures. At pH 7·5 ($-k_D$ 5.34 day^{-1}) or above, a considerable proportion of the apparent accelerated vinclozolin loss could be accounted for by abiotic hydrolysis. At pH values of 5·2 or below (where abiotic degradation is negligible) the rate of vinclozolin degradation by mixed enrichment cultures,[5] and by strain St-1, was less than half that at the pH optimum but this could be partly attributed to the effect of pH on the general growth rate of *P. putida* St-1 irrespective of the nutrient source. In MSSE medium containing 0·1 mM vinclozolin, adjusted initially to pH 6·5, there was virtually no change (within ± 0·1 pH unit) in culture pH during the growth of strain St-1.

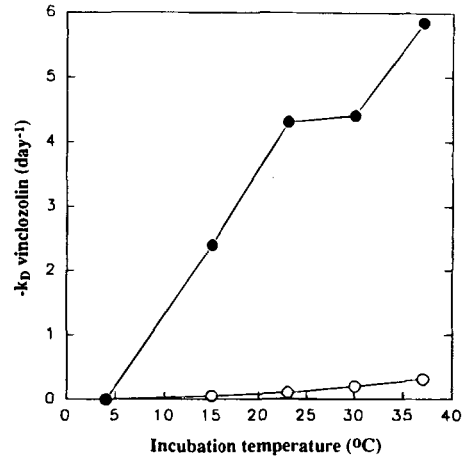

Fig. 6. The effect of incubation temperature on the degradation constant ($-k_D$), for 0·05 mM vinclozolin in MSSE medium, pH 6.5, in (●) cultures of *Pseudomonas putida* St-1 and (○) in uninoculated MSSE medium at 23°C, pH 6·5.

Additions of other sources of carbon, nitrogen or both to soil caused only marginal effects on enhanced degradation of the fungicide.[10] Additions of alternative carbon and nitrogen sources to the mineral salts liquid medium similarly had little effect (less than 10% difference) on rates of vinclozolin degradation by the original mixed cultures or by pure cultures of strain St-1.

3.4 Utilisation of dicarboximide analogues by *Pseudomonas putida* St-1

Cell suspensions (A_{540} approx. 2) of *P. putida* St-1, harvested in exponential phase from MSSE medium containing vinclozolin (Section 2·4), were capable of metabolising only formulated or technical vinclozolin (**1**) (Table 1). The related dicarboximide fungicides, iprodione (**3**) and procymidone (**6**) were not metabolised by this strain nor by strain F. The structurally very similar compound myclozolin (**4**), which differs from vinclozolin only in having a methoxymethyl (CH_3OCH_2-) rather than a vinyl (CH_2CH-) substituent at C-5 of the oxazolidine-2,4-dione ring, was also not metabolised.

3.5 Microbial amendments to soil and their effect on vinclozolin degradation

P. putida St-1, added at 10^8 cells g^{-1} soil to dishes of pretreated soil which already displayed rapid vinclozolin degradation, caused no significant change in the existing rate of vinclozolin disappearance. When added to dishes of previously untreated Close House soil displaying no enhanced degradation of vinclozolin (Fig. 2), however, a vinclozolin supplement was now degraded rapidly with over 90% of the applied fungicide removed in four days (Fig. 7). In untreated soil to which no organisms (but only mineral salts solution) were added, amounts of vinclozolin remained at over 80% of their original applied dose after nine days. No increase in the rates of vinclozolin degradation above that control were

TABLE 1
Utilisation of Dicarboximide Fungicides by Washed Suspensions of *Pseudomonas putida* St-1 Grown on Vinclozolin

Dicarboximide substrate (*0·25 mM AI*)	*Degradation constant* ($-k_D$, day^{-1})[a]
Vinclozolin (formulated)	0·670 (0·030)
Vinclozolin (technical)	0·671 (0·027)
Iprodione (formulated)	0·011 (0·005)
Iprodione (technical)	0·003 (0·015)
Procymidone (formulated)	0·005 (0·001)
Myclozolin (formulated)	0·009 (0·006)

[a] Figures in parentheses are the degradation constants for incubations with identical cell suspensions that were boiled.

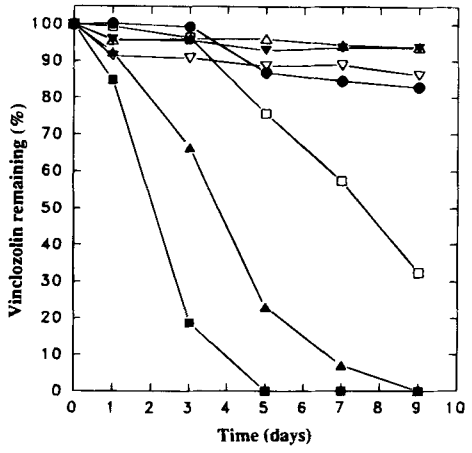

Fig. 7. Vinclozolin degradation in previously untreated soil amended with vinclozolin and a washed suspension of vinclozolin-degrading *Pseudomonas putida* St-1 grown in the presence of the fungicide. Concentrations of residual vinclozolin in soil with: (▽) no cells added; (▼) 10^5 viable cells g^{-1} dry weight of soil; (□) 10^6 viable cells g^{-1} dry weight of soil; (▲) 10^7 viable cells g^{-1} dry weight of soil; (■) 10^8 viable cells g^{-1} dry weight of soil and (●) 10^8 boiled cells g^{-1} dry weight of soil. (△) Dishes of untreated soil amended with 10^8 viable cells g^{-1} dry weight of soil of the same organism previously grown in the absence of vinclozolin.

observed within nine days in soil dishes to which 10^3–10^5 cells g^{-1} dry weight of soil had been added, but with an amendment of 10^6 or 10^7 cells g^{-1} dry weight of soil, stimulation of vinclozolin degradation rates was observed (90% disappearance of vinclozolin required >10 and 6·2 days, respectively). Maximum degradation rates were achieved by the addition of 10^8 cells of *P. putida* St-1 g^{-1} dry weight of previously untreated soil (90% disappearance of vinclozolin in four days) (Fig. 7); cell suspensions more dense than 10^8 cells g^{-1} dry weight of soil (10^9 and 5×10^9 were examined; data not shown) produced no further increase in the rate of vinclozolin degradation. Similarly, soil to which 10^8 boiled cells of vinclozolin-grown *P. putida* St-1 g^{-1} dry weight of soil were added, showed only a marginal increase in vinclozolin degradation rate above that of the unamended control.

When suspensions (10^6–10^8 cells g^{-1} dry weight of soil) of *P. putida* St-1, derived from cultures grown in the absence of vinclozolin, were added to six dishes of untreated soil, a vinclozolin supplement to that soil remained virtually unchanged for 10–12 days (that is, equivalent to the addition of boiled cells or no cells to this soil) (Fig. 7). Subsequent addition of 10^8 cells of the original suspension of vinclozolin-grown *P. putida* St-1 g^{-1} of soil at day 6 to three of these already-amended soil dishes, now led to the immediate onset of vinclozolin degradation which was complete within five days whereas the three remaining dishes, from which this additional supplement of vinclozolin-grown cells was omitted, continued to show no vinclozolin degradation for another five to seven days.

4 DISCUSSION

A simple observation of differences between the rates of pesticide degradation in a pretreated and an untreated soil is, by itself, insufficient evidence to substantiate a claim for enhanced degradation; for a convincing confirmation the microbiology of the phenomenon must be clarified.[18–21] In this paper we have attempted that task for the fungicide vinclozolin. Previous reports of the involvement of a microbial agent in the enhanced degradation of vinclozolin in soil,[5,9,22] have now been substantiated by experiments involving soil sterilization with biocides and microwaves and by the inhibition of enhanced degradation of antibiotics active against prokaryotes, but not fungi.[10] They are confirmed here by the isolation and identification of a vinclozolin-degrading bacterium which imparted enhanced degradation characteristics when added back to a soil which had never received vinclozolin treatments (Fig. 7).

Its morphology, staining characteristics, motility, pigment production in King's medium, absence of growth at 42°C, biochemical activities[17] and a semi-quantitative estimate of the guanosine + cytosine (moles % G + C) content of its DNA[16] showed the isolate to have the characteristics of a pseudomonas and in particular of *Pseudomonas putida* (Trevisan) Migula. This identity is commensurate with the ubiquity of this species in temperate and sub-tropical soils,[17] its well-recognised nutritional versatility[17,25] and its catabolic activity towards a wide range of organic molecules, including many xenobiotics.[17,23–26] There was no biodegradation of vinclozolin at 42°C, consistent with the failure of *P. putida* St-1 to grow at this temperature, and no sign of fungicide degradation in pure or mixed cultures inoculated and incubated at 4°C, though in soil experiments measurable degradation of vinclozolin at 0–4°C had been observed.[10] We recognise that *P. putida* St-1 may be only one of several bacteria (some of which may be psychrophilic) with vinclozolin-degrading abilities in soils showing enhanced degradation, where co-metabolism[21,27] as well as growth-supporting catabolism, of the compound could be a factor contributing to its disappearance.

Enrichments of pH values below 5·5 in our experiments showed minimal removal of vinclozolin and produced no organisms capable of fungicide degradation. A pH of 6·5 was the most successful for elective culture of vinclozolin-degrading micro-organisms and for biodegradation of the fungicide in pure cultures. In liquid cultures, abiotic and biotic transformations of vinclozolin were pH-dependent, which correlates with the recorded pH-dependence of chemical hydrolysis of the fungicide in aqueous buffers.[15,28,29] At pH values above 8·0, vinclozolin (**1**) is in equilibrium with its derivative (**2**) formed by the hydrolytic (1,2) cleavage of the oxazolidine ring; (**2**) or its *N*-decarboxylated analogue may well be the transient intermediate, still containing an aromatic ring, observed in wine[30] and in chicken liver[31] contaminated with vinclozolin, and in bacterial mixed cultures[5] growing at the expense of the fungicide. In contrast the subsequent and persistent metabolite, 3,5-DCA (**5**), was produced abiotically in small amounts only after prolonged incubation at pH values above 8·0.[9,28] Its formation from vinclozolin between 20 h and 90 h in cultures at pH 6·5 and 23°C (Fig. 5(a)) was clearly due to the bacteria because the addition of a biocide or removal of the organisms by filtration or centrifugation caused an immediate cessation of further 3,5-DCA production.

Enumeration of vinclozolin-degrading organisms in liquid culture indicated that 0·25 mM (71·4 $\mu g\ ml^{-1}$) vinclozolin supported an average cell density of $2{\cdot}2 \times 10^7$ cells ml^{-1} (Fig. 3). The estimated commercial field application rate and the application rate of vinclozolin to soil adopted in previous laboratory experiments[10] was 25 $\mu g\ g^{-1}$ dry weight of soil, which from the above principle should be able to support a maximum of $7{\cdot}7 \times 10^6$ cells g^{-1} dry weight of soil, if other nutrient levels remained non-limiting. Total viable counting procedures indicated that the average total cultivable heterotrophic soil population size was $1{\cdot}9 \times 10^9$ cells g^{-1} of soil (similar to that recorded elsewhere[32]) so an increase in population of $7{\cdot}7 \times 10^6$ cells g^{-1} soil, due to vinclozolin availability, represents a 0·4% increase in total cell numbers. Twenty-five micrograms of vinclozolin supplies only 6·3 μg of available carbon, because of the stoichiometric conversion in cultures (Fig. 5(a)) to the 3,5-DCA metabolite which *P. putida* St-1 does not degrade further.[9,33] Assuming a biomass of $6{\cdot}67 \times 10^8$ cells mg^{-1} and that micro-organisms are 50% carbon,[24,34] 25 μg of vinclozolin, supplying 6·3 μg of available carbon, all of which is utilized, should support approximately $8{\cdot}4 \times 10^6$ cells. This value agrees closely with the number of cells (10^7 cells g^{-1} soil) required by untreated soil to exhibit the immediate onset of maximum vinclozolin degradation rates (Fig. 7). It also correlates with the population ($5{\cdot}2 \times 10^6$ cells ml^{-1}) supported in culture experiments (Section 3.3) by 0·05 mM vinclozolin (supplying 3·6 $\mu g\ ml^{-1}$ of available carbon), the theoretical population from which would be $5{\cdot}7 \times 10^6$ cells ml^{-1}. These figures, even with the assumptions made, suggest that most, if not all six, carbons of the non-aromatic moiety of vinclozolin (**1**) may be assimilated into biomass by *P. putida* St-1 and are thus growth-supporting.[27]

Experiments with carbofuran, in contrast, indicated an increase in soil microbial population of only 0·05% from an application of 100 μg carbofuran g^{-1} soil,[35] but in that example only the carbamate side chain was degraded by the micro-organisms and much of the available carbon in it would have been lost as the CO_2 arising from carbamate degradation. In a similar study, the 3- to 15-fold difference in numbers of carbofuran-degrading organisms between untreated and

carbofuran-pretreated soils was still considered insufficient to bring about the rapid degradation of the carbofuran (100 μg g^{-1} soil) applied in the experiments.[36] In the case of isofenphos, too, soils pretreated with 100 μg insecticide g^{-1} soil revealed only 6000–12 000 isofenphos-degrading bacteria g^{-1} of soil,[20] a remarkably small proportion (approx 0·0001%) of the total (cultivable) heterotrophic soil microflora which is usually measured between 10^9 and 10^{10} organisms g^{-1} of soil.[20,32] The biodegradation of these two compounds in soil may thus have an appreciable cometabolic component.[27]

P. putida St-1, indigenous to a field soil exhibiting enhanced degradation of vinclozolin, and capable of growing at the expense of vinclozolin in pure culture (Fig. 3), was able to impart enhanced degradation characteristics to a similar but previously untreated soil when added to it at 10^7–10^8 cells g^{-1} dry weight of soil (Fig. 7). It can thus clearly function in the environment from which it was originally isolated and over a time scale comparable with that observed in field soils brought into the laboratory (Fig. 2). Because, after inoculation into a variety of soils, only slow rates of loss of viability (<2% loss over the first 10 days) have been observed for pseudomonads indigenous to the soils from which they had been obtained[37,38] and because their survival is still evident after three months,[39] it is likely that very few of the organisms added to the soil in the present experiments would have died during the time in which most of the vinclozolin was degraded (three to five days), indicating that the number of organisms added to untreated soil (Fig. 6) to achieve the maximum degradation rates (i.e. 10^7 to 10^8 cells g^{-1} dry weight of soil), approximates the number actually functioning in the soil during the experiment.

We have argued that it is unlikely that only a few original specialist vinclozolin-degrading organisms could attain the population size of 10^7 cells g^{-1} of soil necessary to account for vinclozolin degradation in six to eight days when the experimentally determined generation time on vinclozolin in culture, under optimum conditions, is about 51 h; only three or four doublings would be possible in six to eight days.[10] This doubling time and the cell population it will support is, however, more than adequate to account for the experimentally observed times for disappearance of subsequent test amounts of fungicide:

(i) when small amounts of pretreated soil are mixed with a bulk of untreated soil or
(ii) following pretreatment with very small concentrations of the fungicide.[10]

The alternative explanation, i.e. induction by vinclozolin of catabolic enzymes (in this instance a lactone hydrolase (EC 3.1.1.-)[26,40] and an aryl acylamidohydrolase (EC 3.5.1.3)[41,42] would be involved) in a significant number of existing versatile soil bacteria (c.f. van der Meer *et al.*[43] and references therein) is suggested by the results of Fig. 7 where *P. putida*, cultured in the absence of vinclozolin and returned to untreated soil, did not confer enhanced degradation to it, but where the return of bacteria cultured in the presence of the fungicide did. The oxazole moiety of vinclozolin, although rare among natural products, is known to occur in, e.g., the olivanic acids of *Streptomyces olivaceus* (Waksman) Waksman & Henrici, so appropriate enzymes for its degradation could have already evolved, especially as the first step involves a simple lactone hydrolysis.[29]

It is clear, from the examples quoted, that the transition to enhanced degradation is coupled to significant changes in the catabolic abilities of soil microorganisms but probably to only a modest increase in their numbers,[27,35,43–45] which nevertheless results in a decreased lag period for pesticide degradation and the ability of the enhanced population to maintain this function. Such novel catabolic features can often be conferred by the acquisition by soil bacteria of a 'degradative' (catabolic) plasmid,[23,43,46–48] encoding, in the present instance, the genes for initial steps in vinclozolin degradation. The instability of vinclozolin degradation in *P. putida* St-1 and some of the other original isolates is characteristic, and was originally suspected to be the result, of stresses leading to loss of such a 'degradative' plasmid.[49,50] Plasmid involvement in their biodegradation has been demonstrated for numerous pesticides including 2,4-D,[51] parathion,[52] butylate,[45] EPTC,[6] phenylcarbamates,[53] chloridazon[54] and carbofuran.[5,55–57] Classical plasmid-curing procedures[58] were used to examine whether one of the three endogenous plasmids in our isolated *P. putida* strain was essential for the bacterium to grow at the expense of vinclozolin.[5,59] Phenotypic testing of cured (non-degrading) and wild-type strains revealed that the cured strains could no longer metabolise vinclozolin, but, using published methods,[60] no change in plasmid profile in the molecular mass range of 2 MD–300 MD was observed between the wild-type and the cured derivatives of *P. putida* St-1.[59] There is thus no direct evidence at present that the vinclozolin-degradation trait was plasmid-encoded, though its extreme instability in the isolated bacterium remains strongly suggestive.

Note added in proof

During microbial degradation of 0.7 mM (200 mg l^{-1}) vinclozolin as sole source of carbon and energy by pure cultures of *Bacillus brevis*, *B. cereus* and *Pseudomonas fluorescens*, originally isolated by enrichment from several Russian soils,[61] the pseudomonad transformed some 90% of the available fungicide in liquid mineral

salts medium in 10 days. The principal metabolites were a mixture of *N*-(2-hydroxy-2-methylbut-3-enoyl)-3,5-dichloroanilide, the *N*-decarboxylated derivative of metabolite 2 (Fig. 1) derived by hydrolysis of the 1,2 (i.e. lactone) bond of the oxazolidine ring (42.7%) and of the 1-methyl-1-carboxyprop-2-enyl ester of *N*-(3,5-dichlorophenyl) carbamic acid, the product of the alternative 3,4-bond cleavage of the oxazolidine ring of vinclozolin (38.4%), thus indicating that both lactone and amide bonds may be cleaved simultaneously by a competent bacterium. Unlike the *P. putida* strain described in the present paper, only 3.5% of the original vinclozolin appeared as 3,5-DCA. Moreover, 6–17% of an initial dose of vinclozolin to these Russian soils was still present after a year, whereas previously untreated soils in our field and laboratory studies showed negligible vinclozolin residues after 2 months.

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